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13. ABSTRACT *(Maximum 200 Words)*

Recently, identification of tumor-associated antigens (TAAs) have led to an increased interest in vaccination strategies to treat cancer and/or prevent cancer relapse. Immunotherapy can be used as an adjuvant with other systemic therapy to target micro-metastatic disease and prevent cancer recurrence. Although preclinical/clinical trials using identified tumor-associated antigen for tumor vaccination are ongoing in melanoma and other cancers, breast cancer immunotherapy is limited, due to the limited numbers of breast tumor-associated antigens identified to date. Previously, three candidate breast tumor-associated antigens were identified in our lab by serological analysis of recombinant cDNA expression libraries (SEREX) derived from primary breast tumor and breast cancer cell lines (2-1-1, 7-3 and S3.2). Initial characterization of these candidates by northern blot analysis revealed that 2-1-1 expression is tumor-specific while S3.2 clone is over-expressed in tumor. Although the expression of clone 7-3 is not restricted, its immunoreactivity is positive in high proportion of breast cancer patient sera (7/14). The evaluation of these putative tumor-antigens at the molecular level as well as further evaluation of their immunogenicity may provide insight in breast tumor biology as well as additional targets for breast cancer immunotherapy.

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Table of Contents

Hampton TA, Conry RM, Khazaeli MB, Shaw DR, Curiel DT, LoBuglio AF, Strong TV. SEREX analysis for tumor antigen identification in a mouse model of adenocarcinoma. Cancer Gene Ther 7:446-455, 2000.

Introduction

The recent identification and characterization of tumor-associated antigens (TAAs) has led to increased interest in vaccination strategies to treat cancer and/or prevent cancer relapse. Animal models suggest that these tumor-associated antigens, when presented properly by antigen-presenting cells can break host's tolerance toward its tumor and induce specific antitumor immunity, which results in tumor rejection. Immunotherapy can be used as an adjuvant with other systemic therapy to target micro-metastatic disease and prevent cancer recurrence. While the preclinical/clinical trials focusing on using identified tumor-associated antigen for tumor vaccination are ongoing in melanoma and other cancers, breast cancer immunotherapy is limited, in part due to the limited numbers of breast tumor-associated antigens identified to date. In previous work supported by this grant, three candidate breast tumor-associated antigens were identified by serological analysis of recombinant cDNA expression libraries (SEREX) derived from primary breast tumor and breast cancer cell lines (2-1-1, 7-3 and S3.2). The initial characterization of these candidates by northern blot analysis revealed that 2-1-1 expression is tumor-specific while S3.2 clone is over-expressed in tumor. Although the expression of clone 7-3 is not restricted, its immunoreactivity is positive in high proportion of breast cancer patient sera (7/14). These three clones are not reactive with normal human serum ($n \geq 22$). The evaluation of these putative tumor-antigens has included the molecular analysis of their expression patterns in panels of normal and tumor tissue at the mRNA level. Additional studies will focus on determination of their immunogenicity. The potential of these putative tumor-antigens as targets for immunotherapy may provide insight in breast tumor biology as well as additional targets for breast cancer immunotherapy.

Statement of Work (Revised 11/99)

Task 1: Complete molecular characterization of candidate tumor antigens:

complete sequence analysis of tumor antigens (specifically clone 2-1-1) (80% complete)
complete expression analysis by Northern blot in tumor and normal tissue (complete)
complete studies of reactivity of antigens with allogeneic patient sera (partially complete)
complete mutational analysis (partially complete)

Task 2 Assess immunogenicity of putative tumor antigens: (initiated)

produce RNA
transfect dendritic cells and assay for ability to induce primary immune response in autologous T cells in healthy individuals and individuals with breast cancer

BODY

1. Three candidate human breast tumor antigens have been identified in our laboratory by SEREX analysis.

Previously, three candidate antigens were identified and the clones were named 2-1-1, 7-3 and S3.2. For the identification of **2-1-1** and **7-3 clone**, a recombinant cDNA expression library was constructed from four breast cancer cell lines: MCF-7, MDA-MB453, T47D, and ZR75-1 (kindly provided by J.M. Ruppert) and was immunoscreened for reactivity with high-titered IgG antibodies presented in serum from six individuals with advanced breast cancer. For the identification of **clone S3.2**, a cDNA library was constructed from tumor of a patient with advanced breast cancer and immunoscreened with autologous serum. The reactive plaques were purified, restriction digested to identify clonal similarity. The identified clones were then submitted to in vivo excision of pBluescript phagemids into plasmid form for further characterization.

2. Initial characterization of these candidate antigens has been accomplished.

2.1 Seroactivity analysis

The frequency of antibody response to candidate antigens was examined among normal and patient sera. Briefly, phage containing three individual inserts were mixed 50/50 with no-insert vector as negative control and plated. After a 4-hour incubation at 42°C, protein expression was induced by incubating plates with isopropyl β-D-thiogalactoside (IPTG) for an additional 4 hours. Filters were lifted, blocked and incubated with sera from normal individuals and patients with breast, ovarian or head and neck carcinoma. Sera was preabsorbed with E.coli phage lysate and diluted to 1:250. Alkaline phosphatase conjugated goat anti-human antibodies (Jackson laboratory) and an NBT/BCIP colorimetric assay was used to detect positive reactions.

From the preliminary data we found that these three putative breast tumor antigens are promising in that reactivity with normal human sera has not yet been detected. Interestingly, clone 7-3 is reactive with the large proportion of breast cancer patients (7/14), suggesting that reactivity with the antigen may be common, and perhaps specific for breast cancer patients. Additional studies will be needed. The seroactivity of these three clones are summarized in table1.

Table 1. IgG antibody reactivity with putative breast tumor antigens

Clone Name	Breast Cancer Sera	Ovarian Cancer Sera	Head & Neck Cancer Sera	Normal
7-3	7 of 14	0 of 7	0 of 7	0 of 22
2-1-1	2 of 16	0 of 10	n/d	0 of 25
S3-2	2 of 30	0 of 3	0 of 7	0 of 30

Sera from individuals with carcinoma of the breast, ovary, or head and neck were evaluated for reactivity with the candidate antigens. None of the clones was reactive with normal human sera. The number of positive serum samples and the total number tested are given. Positive samples were reactive with the plaque encoding the putative breast tumor antigens at a dilution of 1:250.

2.2 Sequence analysis of identified clones.

Restriction enzyme mapping and 5' and 3' sequencing revealed that these clones had inserts of different sizes ranging from approximately 1.3 to 3.0 kb. Sequencing analysis of cDNA inserts was carried out by automatic thermal cycle sequencing machine at core facility center at UAB using the T3 and T7 primers in the vector. Specific internal oligonucleotides were designed as the sequencing progressed. Sequence alignment was performed with BLAST search (homology search run through the National Center for Biotechnology Information).

The **S3.2 clone** was found to have homology with several described expressed sequence tags (ESTs). Several short open reading frames were identified. However the relevant ORF and epitope(s) have not yet been determined. Partial sequence analysis of **7.3 clone** revealed it to be the apparent human homologue of mouse Mem3 gene. Both 5' and 3' ends of the insert exhibit strong homology with this gene, and the insert is of the approximate size, suggesting that the isolated clone encodes the entire gene. This gene is reported to be expressed at high levels in the mouse embryos and at low levels in adult tissues. Its exact function is not known, but it shares homology with a yeast sorting protein. To date, approximately 80% of **2-1-1 clone** has been sequenced. Sequence homology as determined by BLAST search revealed that the insert has strong homology (99%) to the N-terminal domain of human M-phase phosphoprotein1, a cell cycle protein involved in disassembly of interphase structures and generation of M phase enzymatic activities and structures (figure 1).

2.3 Analysis of expression pattern

The expression of these genes was analyzed by northern blot hybridization using 10 microgram of total RNA from panels of normal tissues, primary breast tumors, breast cancer, ovarian cancer and melanoma cell lines blotted onto nylon membranes.

For **S3.2 insert**, we detected a moderate expression of 1.3 kb transcript in normal white blood cell, fibroblast, liver, ovarian and oral tissue. We detected the high expression of the same transcript size in 4/4 primary breast tumor, 2/2 melanoma, and 3/3 breast cancer cell lines. Thus, this transcript appears to be overexpressed in tumor cells. Figure 2

For **clone 2-1-1**, no expression was detected in the panel of normal tissues mentioned above. Weak expression signal of approximately 6 kb transcript was detected in 2/4 primary breast tumors, 2/2 melanoma cell lines. Moderate signals were detected in 4/4 breast cancer cell lines and 4/4 ovarian cancer cell lines. Thus far, the transcript appears to be specifically expressed in tumor cells. Given that 3.0 kb insert demonstrated strong homology to a portion of human M-phase phosphoprotein1 (MPP1) cDNA and its transcript size also correlate with the size of MPP1 cDNA, this clone apparently represent MPP1. Figure 2

For **7.3 clone,** northern blot analysis showed moderate expression of 2.3 kb transcript in normal breast epithelium and 4 ovarian cancer cell lines and 4 breast cancer cell lines that were used to construct the cDNA libraries in this experiment. Four additional breast tumors showed similar high-level expression (not shown).

In summary, we have identified three candidate breast tumor-associated antigens. Initial molecular characterization demonstrates favorable characteristic (expression pattern, seroreactivity) consistent with tumor antigens. Our plan is to continue with the molecular characterization of these antigens. We will also perform more extensive analysis of the immune response elicited by these antigens and the relevance of that immune response to breast cancer. These studies will allow us to determine if these proteins may be useful for diagnostic testing, staging and/or for immunotherapy of this disease.

Figures

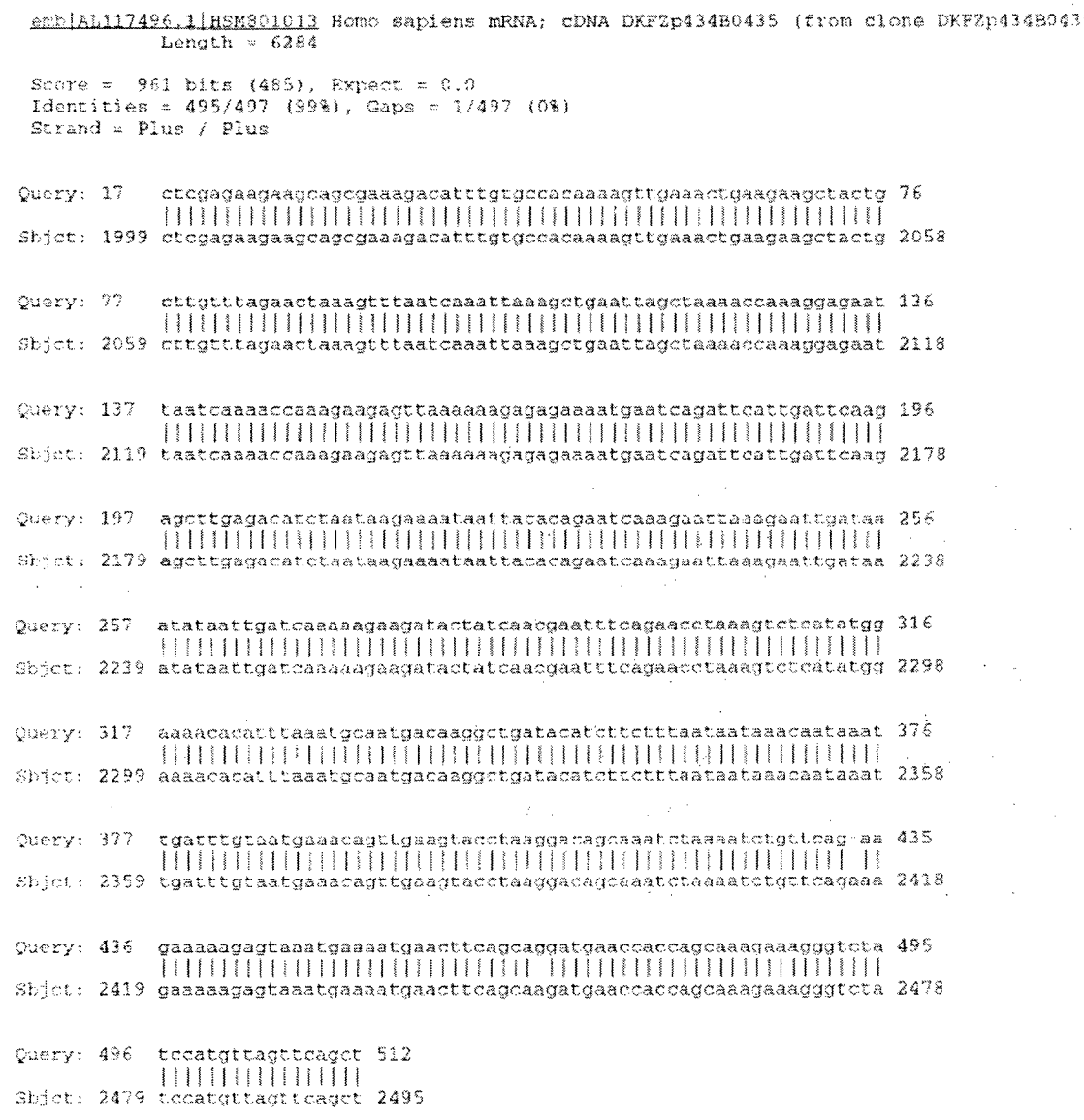
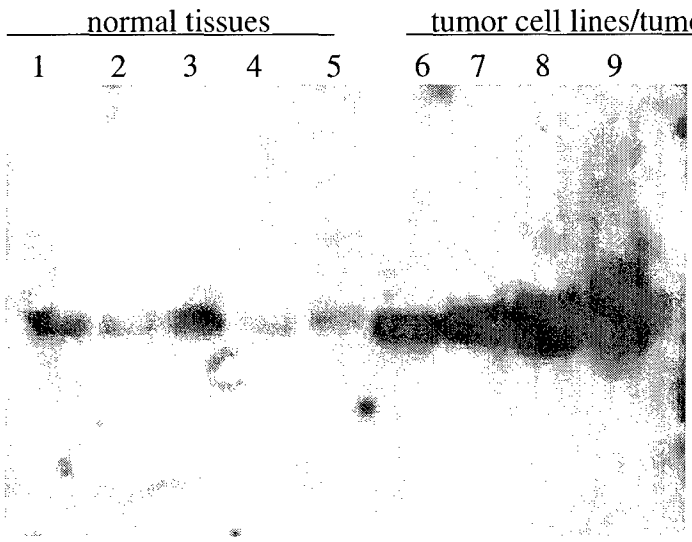


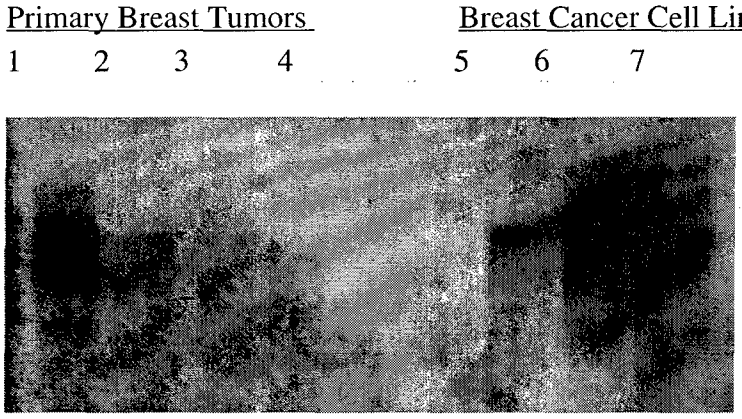
Figure 1. Alignment of clone 2-1-1(Query) with human M-phase phosphoprotein-1 cDNA(Sbjct). This alignment shows the high degree of homology between the two genes. The size of 2-1-1 transcript is also correlated with MPP-1 full length cDNA, suggesting that 2-1-1 clone represents MPP-1.

Expression of S3.2 in normal tissue, tumor cell lines, and a primary breast tumor



1. liver
2. ovary
3. oral epithelium
4. white blood cells
5. fibroblast
6. Melanoma 624
7. Melanoma 888
8. MDA-MB-453 (breast)
9. Primary Breast tumor 1

Expression of 2-1-1 in primary breast tumors and breast cancer cell lines



1. Breast tumor 1
2. Breast tumor 2
3. Breast tumor 3
4. Breast tumor 4
5. ZR 75
6. T47D
7. MDA-MB-453

Figure 2. Northern blot analysis of S3.2 and 2-1-1 in RNA from normal tissues (liver, ovary, oral cavity, white blood cells, fibroblast), tumor cell lines (melanoma 624, melanoma 888, breast MDA-MB-453) and primary breast tumor. 3a) S3.2 probe recognizes a transcript of approximately 1.3 kb in size Moderate level of expression were detected in normal tissues, while high level expression were found in tumor cell lines and primary breast tumor tissues. Four additional breast tumors show similar, high level expression (not shown). 3b) 2-1-1 probe recognizes a transcript of approximately 6 kb in size. Northern blot analysis revealed expression in 2 of 4 primary breast tumors, as well as ovarian and melanoma cell lines. Expression in normal tissue was absent (not shown).

Key Research Accomplishments

1. Serelogical studies revealed promising immunoreactivity for all three clones. Clone 7-3 is highly reactive with 7/14 breast cancer patient sera.

2. Sequence analysis of the three clones further characterized the putitive tumor antigens as follows:

Clone S3.2 has homology with several ESTs, yet the ORF has yet to be determined.

Clone 7.3 appears to be the human homologue of the murine Mem3 gene which is highly expressed in murine embyros but at low levels in adults.

80% of clone 2-1-1 has been sequenced and it shows 99% homology to human M-phase phosphoprotein which is a cell cycle protein involved in disassembly of interphase structures and the generation of the m phase.

3. Expression analysis is summarized in Table 2

Table 2. Expression Analysis. This table shows the results of Northern blot analysis.

Clone	Tissue Expression	Level of Expression
S3.2	White blood cells, fibroblasts, liver, ovarian and oral tissue.	Moderate
	4/4 primary breast tumors, 2/2 melanoma tumors and 3/3 breast cancer cell lines.	High
2-1-1	White blood cells, fibroblasts, liver ovarian and oral tissue.	None
	4/4 breast cancer cell lines, 4/4 ovarian tumor cell lines.	Moderate
	2/4 primary breast tumors, 2/2 melanoma cell lines.	High

7.3	4/4 breast tumors.	High
	Normal brest epithelium, 4/4 ovarian cancer cell lines, and 4/4 breast cancer cell lines.	Moderate

Reportable Outcomes

Manuscripts, Abstracts and Presentations

1. Manuscript:

Hampton TA, Conry RM, Khazaeli MB, Shaw DR, Curiel DT, LoBuglio AF, Strong TV. SEREX analysis for tumor antigen identification in a mouse model of adenocarcinoma. Cancer Gene Ther 7:446-455, 2000

2. Abstracts and Presentations:

Hampton TA, Conry RM, Sumerel L, Khazaeli MB, Curiel DT, LoBuglio AF, Strong TV. Serological identification of the murine endogenous leukemia proviral envelope protein as a tumor antigen in MC38 cells. Cancer Gene Therapy 4, 1997.

Strong TV, Guerrero A, Hampton TA, Conry RM, Ruppert JM, Curiel DT, LoBuglio AF. Serological identification of human breast tumor associated antigens by recombinant expression cloning. American Association of Cancer Researchers 39:262, 1998. (March 1998)

Hampton TA, Conry RM, Sumerel L, Khazaeli MB, Curiel DT, LoBuglio AF, Strong TV. The murine endogenous leukemia proviral envelope protein acts as a tumor antigen in MC38 cells. Gordon Research Conference on Cancer Molecular Biology, August, 1998.

Degree Obtained:

The initial principal investigator, Tracy Hampton, received her Ph.D. based on this work.

Employment/Training

Tracy Hampton received her Ph.D. and received, as a result of this work, a Postdoctoral Fellowship at Stanford University.

Conclusions

Currently potential target antigens for breast cancer immunotherapy are HER-2/neu protein, carcinoembryonic antigen (CEA), MAGE-1, and MUC-1. These antigens are pursued based on their high level expression in breast tumor tissue compared to normal tissue as well as an understanding of epitopes recognized by CTLs. Because each of these antigens is expressed only on a portion of breast tumors, only a portion of breast cancer patients may benefit from therapy directed at these targets. Expanding the list of antigen targets will be of considerable importance in the development of effective immunotherapy strategies. The analysis of the candidate antigens we have identified at DNA, mRNA and protein level in a number of normal tissues, breast tumors and other tumor types will delineate the expression pattern of these antigens and may provide a better understanding of the role(s) of these proteins in breast cancer. It will also be important to assess the population of breast cancer patients who have circulating antibodies against these antigens. This information is crucial for the design of vaccine strategies, because it is necessary to know which proportion of breast cancer patients express the gene and where the gene is normally expressed.

His-tagged fusion proteins have been made from these clones in order to further extend the analysis of the antibody response to these candidate antigens. These His-tag fusion proteins will provide relatively rapid affinity-purification of fusion protein for the generation of polyclonal antibodies which will be used in immunohistochemistry studies and in situ hybridization (ISH). The investigation of gene expression by ISH will help to determine the expression pattern of these transcripts directly in tissue. ISH can also be used for co-evaluation with the results obtained from immunohistochemistry.

The goal is to develop an ELISA-based screening using sera from a large number of cancer patients and healthy individuals. This will allow the determination of the frequency of antibodies against these proteins in different populations. We will increase the number of normal serum to 50 individuals and approximately 50 additional serum samples from breast, ovarian and melanoma cancer patients will be obtained in order to expand the study. DNA sequences on these clones will be completed and compared to public database sequences. For S3.2 whose expression is increased in tumor compared to normal tissue, Southern blot analysis will be performed to identify possible genomic amplification or rearrangement.

The characterization of breast tumor-associated antigens is important for the understanding of genetics, antigenicity and progression of breast tumors. Furthermore, well-characterized tumor associated antigens can be used as targets for cancer immunotherapy to enhance host

tumor-specific immune response. The remaining year of funding will focus on completing molecular characterization of these antigens and evaluating immunogenicity.

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SEREX analysis for tumor antigen identification in a mouse model of adenocarcinoma

Tracy A. Hampton,[1–3] Robert M. Conry,[2] M. B. Khazaeli,[2] Denise R. Shaw,[2] David T. Curiel,[1,2] Albert F. LoBuglio,[2] and Theresa V. Strong[1–3]

[1]Gene Therapy Center, [2]Division of Hematology/Oncology, Department of Medicine, and [3]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294.

Evaluation of immunotherapy strategies in mouse models of carcinoma is hampered by the limited number of known murine tumor antigens (Ags). Although tumor Ags can be identified based on cytotoxic T-cell activation, this approach is not readily accomplished for many tumor types. We applied an alternative strategy based on a humoral immune response, SEREX, to the identification of tumor Ags in the murine colon adenocarcinoma cell line MC38. Immunization of syngeneic C57BL/6 mice with MC38 cells by three different methods induced a protective immune response with concomitant production of anti-MC38 antibodies. Immunoscreening of an MC38-derived expression library resulted in the identification of the endogenous ecotropic leukemia virus envelope (env) protein and the murine ATRX protein as candidate tumor Ags. Northern blot analysis demonstrated high levels of expression of the *env* transcript in MC38 cells and in several other murine tumor cell lines, whereas expression in normal colonic epithelium was absent. *ATRX* was found to be variably expressed in tumor cell lines and in normal tissue. Further analysis of the expressed *env* sequence indicated that it represents a nonmutated tumor Ag. Polynucleotide immunization with DNA encoding the env polypeptide resulted in strong and specific antibody responses to this self Ag in all immunized mice. Thus, SEREX offers a rapid means of identifying tumor Ags in murine cancer models. **Cancer Gene Therapy (2000) 7, 446–455**

Key words: Tumor antigen; endogenous retrovirus; antibody; adenocarcinoma; ATRX.

The concept of cancer immunotherapy is predicated on the existence of tumor-associated antigens (TAAs) against which the host is capable of mounting an immune response, leading to tumor rejection. To this end, immune responses in tumor-bearing individuals have been described for a variety of mutant tumor antigens (Ags), such as mutant p53[1–3] and ras,[4–7] as well as nonmutant tumor proteins including erbB-2,[8,9] MART-1,[10,11] MAGE-1,[12,13] tyrosinase,[14,15] and MUC-1.[16] Such tumor-specific responses suggest that the immune system may be amenable to strategies that further induce or enhance patients' immune responses to their own tumor cells. Important to the concept of developing effective immunotherapies is the ability to break immunological tolerance to self Ags. However, the ability to evaluate approaches for breaking tolerance to specific Ags in mouse models of cancer is limited by the fact that few murine tumor Ags have been described to date. Accordingly, many murine immunotherapy models use heterologous Ags (for example, Refs. 17–19), which may not address issues critical to the development of effective immunotherapeutic strategies. A more complete understanding of the spectrum of murine Ags capable of eliciting antitumoral immune responses is needed to better understand the interaction of cancer and the immune system as well as for the development of more effective cancer vaccines.

Several methods have been employed to isolate and clone TAAs. Due to the critical role of cytotoxic T lymphocytes (CTLs) in mediating antitumoral immunity, these approaches have largely consisted of the isolation of Ags recognized by CTLs that have been expanded *in vitro*. Tumor Ags have been identified in this manner by acid elution of antigenic peptides bound to major histocompatibility complex class I molecules.[20] A second, genetic approach assays CTL responsiveness to target cells transfected with cDNA libraries to identify immunogenic peptides.[21,22] Although these approaches have proven valuable for TAA identification in some tumor types, particularly human melanoma, widespread application is hampered by difficulties in establishing long-term CTL cultures. In addition, it has not yet been established that CTLs generated *in vitro* are representative of the tumor-specific CTLs *in vivo*. Thus, complementary approaches for the identification of novel immunogenic tumor Ags are warranted.

The activation of CTL responses is dependent upon T-helper (Th) cells and may be accompanied by the induction of humoral immunity. Indeed, humoral im-

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mune responses to known TAAs have been described in tumor-bearing individuals.[1,2,23–27] The presence of antibodies (Abs) directed toward known tumor Ags suggests the potential usefulness of serum for the identification of novel TAAs. The feasibility of such a strategy for identifying tumor Ags was demonstrated by Sahin et al,[28] who identified both known and novel tumor Ags using autologous sera from cancer patients to screen tumor-derived expression libraries, an approach they termed "serologic identification of Ags by recombinant expression cloning" (SEREX). This technique has succeeded in greatly expanding the scope of human tumor Ags.[29] By comparison, the catalog of cloned mouse tumor Ags remains quite small.

Mouse models of syngeneic tumors provide a useful system in which to investigate the nature of TAAs. The MC38 colonic adenocarcinoma cell line was derived from C57BL/6 mice treated with the carcinogen 1,2-dimethylhydrazine.[30] These cells produce tumors when injected subcutaneously in syngenic mice, but immunization of naive syngeneic mice with MC38 cells can induce an immune response that mediates tumor rejection upon challenge with an otherwise lethal dose of live cells. Thus, these cells express cryptic tumor Ags that stimulate antitumoral immune responses *in vivo*. Here, we describe the application of SEREX technology toward the identification of MC38 Ags. This approach identified the endogenous ecotropic retroviral envelope (env) and the murine ATRX proteins as putative tumor Ags in these cells. These Ags were reactive with sera from mice immunized with MC38 cells in three different ways. The env protein was found to be a nonmutant tumor Ag, and polynucleotide immunization was used to demonstrate that an immune response could be elicited to this self Ag. These studies demonstrate the utility of SEREX as a means to rapidly identify murine tumor Ags for further evaluation in immunotherapy models.

MATERIALS AND METHODS

Cell lines and culture conditions

MC38 and MC38-CEA-2[31] cells were kindly provided by Dr. Steven Rosenberg (National Cancer Institute, Bethesda, Md) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf sera (FCS). B16 is a mouse melanoma cell line that arose spontaneously in C57BL/6 mice.[32] These cells were maintained in Eagle's minimal essential medium with 10% FCS. The mouse lymphoma cell line EL4,[33] a chemically induced line derived from a C57BL/6 mouse, was maintained in DMEM supplemented with 10% horse sera. The mouse mammary tumor cell lines 4T01 and 4T07 are sublines of a spontaneously arising mammary tumor in a BALB/cfC$_3$H mouse;[34] both of these lines were maintained in DMEM/F12 supplemented with 10% FCS. CT26 cells are colonic adenocarcinoma cells from a BALB/c mouse induced by *N*-nitroso-*N*-methylurethane;[35] they were cultured in RPMI 1640 with 10% FCS. P815, a mastocytoma line derived from a DBA/2 mouse,[36] was maintained in RPMI 1640 with 10% FCS. C2C12, a mouse myoblast cell line (American Type Culture Collection, Manassas, Va), was maintained in DMEM with 10% FCS.

Immunization protocol

To elicit anti-MC38 immune responses, groups of 12 mice were immunized in one of three ways. Group I mice were injected with 5×10^5 MC38 cells into the right foot pad and tumors were excised 10–14 days later. A total of 10 of 12 mice survived the amputation and were available for challenge on day 29 with 3×10^5 cells injected subcutaneously into the flank. A total of 8 of the 10 mice rejected the day 29 tumor challenge and received a second identical tumor challenge on day 79, followed 14 days later by exsanguination to harvest sera. Group II mice were injected subcutaneously on days 1 and 15 with 1×10^6 MC38 cells that had been irradiated with 15,000 cGy. Mice were then challenged with 3×10^5 nonirradiated tumor cells on day 29. A total of 3 of 12 animals developed tumors and were sacrificed. The remaining nine animals were rechallenged with 3×10^5 nonirradiated tumor cells on day 54. Sera were collected from these animals 14 days later. Group III animals were immunized with a plasmid DNA encoding human carcinoembryonic Ag (CEA) and challenged with MC38 cells stably transfected to express human CEA (MC38-CEA-2[31]). We have shown previously that polynucleotide immunization with a CEA-encoding plasmid results in reliable rejection of MC38-CEA-2 cells.[37] Thus, 12 mice received intramuscular injections of 50 μg of plasmid-encoding CEA on days 1 and 15, followed by tumor challenge with 3×10^5 MC38-CEA-2 cells on days 29, 41, and 54. A total of 8 of 12 mice successfully rejected all three challenges with MC38-CEA-2 cells, and sera were collected from these mice 14 days after the last challenge. In each of the three immunization experiments described above, tumors grew in 12 of 12 naive control mice, validating each challenge with MC38 or MC38-CEA-2 tumor cells.

Assay for Ab response to intact tumor cells

To determine whether the various immunization strategies had elicited an Ab response to MC38 tumor cells, a cell-binding assay was performed as described previously.[38] Briefly, tumor cells were washed with phosphate-buffered saline (PBS) and 5×10^5 cells were aliquoted per tube in 100 μL of PBS with 1% bovine serum albumin (PBE). Each tube then received 100 μL of a 1/10 dilution of immune sera or normal mouse sera in PBE, and the cell/serum mixture was incubated at room temperature for 1 hour on an orbital shaker. Cells were then washed with 4 mL of PBS to remove unbound Ab. After washing, the cells were resuspended in 100 μL of PBS containing 0.1% pigskin gelatin and 100 ng of ^{125}I-labeled *Staphylococcus aureus* protein A (SPA) (~200,000 cpm per tube), and incubated for 1 hour at room temperature with shaking. SPA was obtained from Sigma (St. Louis, Mo) and was radiolabeled by the Iodogen method (Pierce, Rockford, Ill). The cells were washed with 4 mL of PBS and counted in a gamma scintillation counter. The specific activity of the ^{125}I-labeled SPA was used to convert counts per minute bound to the number of molecules bound per cell.[38] The SPA binding to cells incubated in PBE only (no sera) was subtracted as nonspecific binding. MC38-CEA-2 cells incubated with CEA-specific monoclonal Ab (COL-1) served as a positive control.

cDNA library construction and immunoscreening

Total RNA from MC38 cells or MC38-CEA-2 cells was isolated using an RNA Stat 60 RNA isolation reagent (Tel-Test B), and mRNA was isolated on oligo(dT) beads (PolyA-Quik mRNA isolation kit, Stratagene, La Jolla, Calif). A cDNA library was constructed in the Zap Express vector (Stratagene), which is capable of directing expression in both

prokaryotic and eukaryotic hosts. Briefly, mRNA was reverse transcribed using an oligo(dT) primer with an internal *Xho*I site as well as a random primers that included an *Xho*I restriction site at the end. The reverse transcription reaction included 5-methyl deoxycytidine triphosphate. After second-strand synthesis, *Eco*RI adapters were added by ligation and the cDNA was restricted with *Xho*I. cDNA fragments were cloned into the directional λZap Express vector, packaged, and used to infect *Escherichia coli* cells. As a preliminary characterization of the library, inserts from random plaques were amplified by polymerase chain reaction (PCR) and demonstrated insert sizes ranging from ~300 bp to 3.5 kb with >90% recombinants (data not shown).

For immunoscreening, recombinant plaques were plated at a density of ~20,000 plaques per 150-mm plate, and protein expression was induced using nitrocellulose filters saturated with isopropyl β-D-thiogalactoside. Filters were lifted, blocked with 1% bovine serum albumin in Tris-buffered saline (20 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5) and 150 mM NaCl), and screened with sera from mice immunized as described above to detect reactive plaques. Primary mouse sera were preabsorbed with *E. coli* phage lysate (Stratagene) and diluted 1/250 for screening. After incubating filters with diluted mouse sera, the filters were washed with Tris-buffered saline with 0.05% Tween 20 (Sigma) and incubated with alkaline phosphate-conjugated goat anti-mouse immunoglobulin G (IgG) Abs (Stratagene) at a dilution of 1/5000 for 1 hour at room temperature. After washing, a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colorimetric substrate was used to identify positive clones. Positive plaques were purified to clonality for further study.

DNA sequencing

Phagemid DNA from positive plaques was rescued by *in vivo* excision using a helper phage system (Stratagene). Purified DNA was sequenced by dideoxy sequencing using Sequenase 2.0 (United States Biochemical, Cleveland, Ohio) and ^{35}S-labeled deoxyadenosine triphosphate. Approximately 250 bp of sequence were determined at both the 5′ and 3′ ends using T3 and T7 primers, respectively. Sequences were searched against the National Center for Biotechnology databases using the BLASTN program.

To sequence the *env* gene expressed in MC38 for comparison with C57BL/6 genomic sequences, PCR products were generated from reverse-transcribed MC38 total RNA and C57BL/6 genomic DNA. The 2.6-kb coding region was amplified using the following primers: 5′-GAGAA*AAGCTT*CGCCCCCGATAAACCATGGAG-3′ and 5′-GCGC*AAGCTT*TGCACCAGCAAAAGGCTTTATT-3′. *Hin*dIII sites are italicized. Sequencing of the PCR products was performed using an ABT automated DNA sequencer (Perkin Elmer Biosystems, Foster City, Calif) with the PCR primers as well as internal sequencing primers.

Northern blot analysis

Total RNA was isolated from cultured cells using RNA Stat-60 (Tel-Test B) according to the manufacturer's directions. To isolate RNA from colonic epithelium, a section of colon was isolated and opened longitudinally; next, the epithelium was recovered by gentle scraping. Epithelial cells were homogenized in RNA Stat-60 and processed as for the cultured cells. Approximately 8 μg of total RNA from each sample was electrophoresed on a 1% denaturing agarose gel and transferred to a nylon membrane (Amersham, Arlington Heights, Ill). The blot was prehybridized for 2 hours at 65°C in a solution of 6× standard saline citrate (SSC), 5× Denhardt's, 100 μg/mL sheared salmon sperm DNA, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS). For analysis of *env* expression, 200 ng of a random-primed ^{32}P-deoxycytidine triphosphate-labeled probe was generated corresponding to bp 6004–6543 of the *env* gene, a *Bgl*II to *Bam*HI fragment. This probe recognizes only the ecotropic *env* sequence.[39,40] For *ATRX*, the 2.8-kb insert was isolated and labeled. Each probe was hybridized overnight in hybridization solution as described above, with the probe at a concentration of 20 ng/mL. After hybridization, the blot was washed successively in 2× SSC with 0.5% SDS, 2× SSC with 0.1% SDS, and 0.1× SSC with 0.1% SDS; next, the blot was autoradiographed at −70°C for a period of 1–4 days. To control for loading differences, the blot was stripped with boiling 0.1% SDS and rehybridized with a probe specific for the mouse β-actin gene (Stratagene).

Southern blot analysis

For Southern blot analysis, genomic DNA from MC38 cells, B16 cells, and normal colonic epithelial cells was isolated by lysis in 50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM NaCl, and 1% SDS, and by treatment with 100 μg/mL Proteinase K overnight at 50°C. After phenol/chloroform extraction, the DNA was precipitated with ethanol and resuspended in 10 mM Tris and 1 mM EDTA (pH 8.0). Approximately 10 μg of DNA was subjected to digestion with the appropriate restriction enzyme, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (Amersham). The membrane was hybridized with an *env*- or an *ATRX*-specific probe as described above.

Polynucleotide immunization and isotype analysis

Groups of 10 mice were immunized with DNA encoding the 1.3-kb partial *env* cDNA or with vector backbone. Intramuscular injections of 50 μg of plasmid DNA (at 1 mg/mL) were made on days 1, 15, and 29. Sera were collected 4 days after the third immunization for analysis of env-specific Ab *via* screening against the env-expressing plaques as described above. For isotype analysis, alkaline phosphatase-conjugated, isotype-specific Abs (Clonotyping System, Southern Biotechnology Associates, Birmingham, Ala), were used as secondary Abs at a dilution of 1/1000.

RESULTS

Immunization and detection of Abs directed against MC38 Ags

Three groups of mice were immunized to generate sera reactive with MC38 cells. Group I mice were injected with MC38 cells with subsequent tumor excision, and group II animals were immunized with irradiated MC38 cells. Both of these groups were then challenged with live MC38 cells, which were rejected by most animals (17 of 22). A third group of animals was immunized with plasmid DNA encoding human CEA (pCEA) and challenged with MC38 cells expressing CEA, as reported previously.[37] A total of 8 of 12 animals in this group rejected three consecutive tumor challenges. After the immunization schedules were completed, the presence of MC38-reactive Abs was detected in an MC38 cell-binding assay (Fig 1). All three groups of immunized

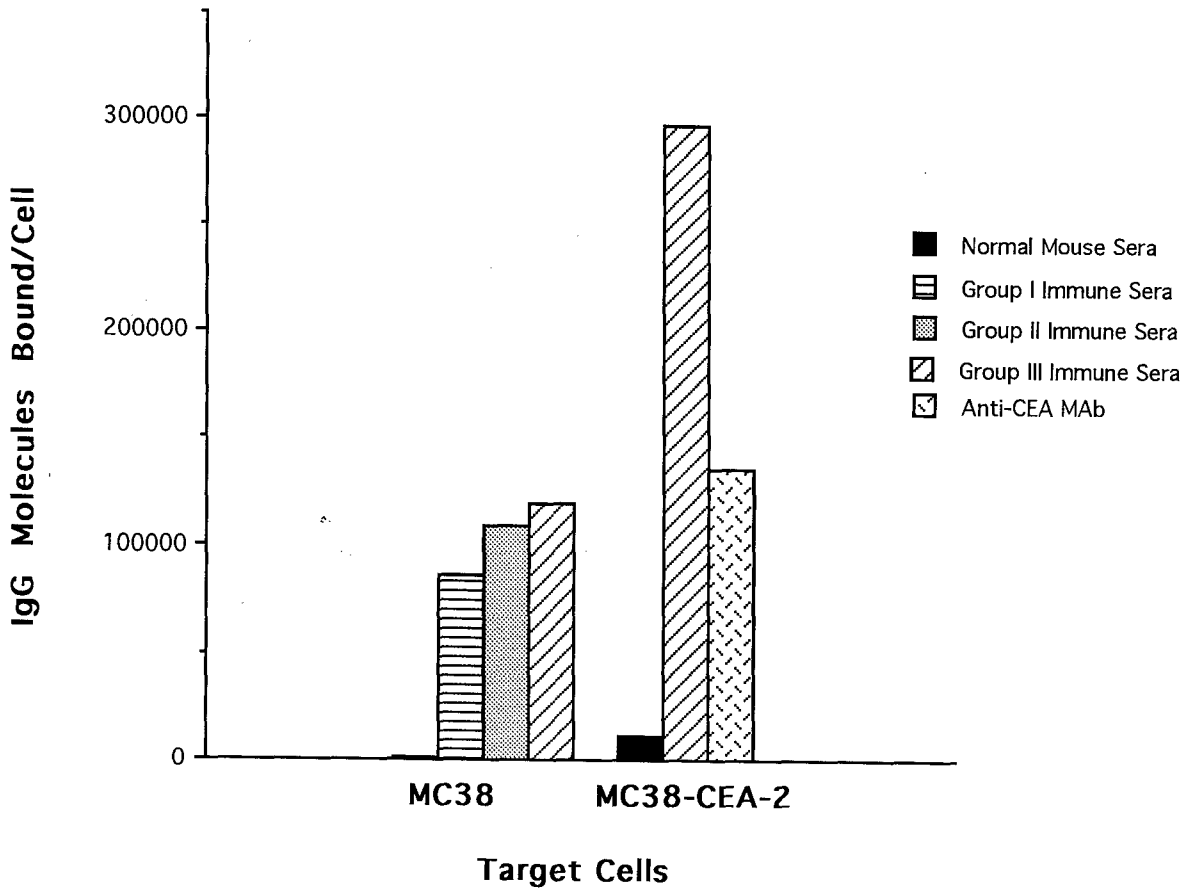


Figure 1. Ab response to intact tumor cells. Sera collected from mice immunized as outlined in *Materials and Methods* were examined for Ab binding to intact tumor cells. Antisera from naive mice or immunized mice (groups I-III) were incubated with MC38 cells. In addition, sera from naive mice and from the animals immunized in group III were incubated with MC38-CEA-2 cells. Results are reported as the number of molecules of Ab bound per cell, as described in *Materials and Methods*. Binding of a CEA-specific monoclonal Ab (COL-1) to MC38 cells stably expressing human CEA (MC38-CEA-2) served as positive control.

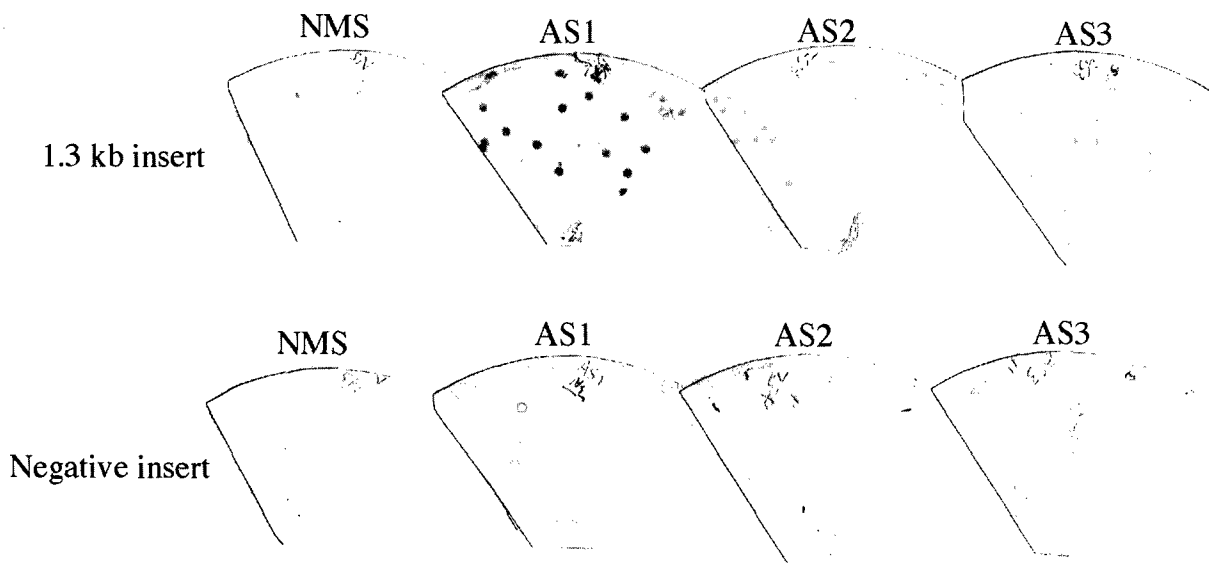
mice demonstrated high levels of Ab binding to intact MC38 cells, whereas naive mouse sera did not. Mice immunized with pCEA and challenged with MC38-CEA-2 cells produced Abs to the parental MC38 cells in addition to CEA-specific Abs, as manifested by increased Ab binding to MC38-CEA-2 cells compared with parental MC38 cells. Thus, these serum samples contained Abs that were useful for detection of cryptic TAAs in MC38 cells.

Identification of reactive plaques

Sera from immunized animals (group III) were used to screen an MC38-derived cDNA library. Reactive plaques were then purified to homogeneity in secondary and tertiary screens. Screening of $\sim 10^6$ plaques resulted in the identification of two independent positive plaques. Amplification of the insert of one of these plaques by PCR demonstrated the presence of a 1.3-kb insert. Importantly, sera from animals immunized with MC38 cells by all three routes described above (groups I, II, and III) were strongly reactive with the 1.3-kb plaque (Fig 2). Isotype analysis revealed that reactive Abs were predominantly IgG2a and IgG2b, with IgM and IgG1 being weakly reactive (data not shown). Dideoxy sequencing was performed on rescued plasmid DNA using the T3 and T7 primers present in the vector. A search of the National Center for Biotechnology database revealed the identity of the clone with a portion of the endogenous ecotropic leukemia proviral sequence (MuLV). This insert sequence includes the 3′ portion of the env protein sequence (*env*) as well as the 3′ long terminal repeat (LTR) (GenBank accession numbers J01998 and U63133) (Fig 2). The clone contains base pairs 7071–8374 of the published sequence,[41] corresponding to amino acid 430 to the C terminus of the env protein. The env polypeptide encoded by this clone includes the last one-third of the extracellular portion of the protein, as well as the entire transmembrane domain. A 99-bp deletion of the 3′ LTR was also noted, as discussed below.

The second plaque had an insert size of ~2.8 kb and was also reactive with all groups of MC38-immunized animals. Sequence analysis of the insert revealed that it represents a portion (base pairs 503-3327) of the murine

A



B

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1.3 kb:     14 CAACCACCATACTTGACCTCACCACCGATTACTGTGTCCTGGTCGAGCTTTGGCCAAGGG 73
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J01998:   7071 CAACCACCATACTTGACCTCACCACCGATTACTGTGTCCTGGTCGAGCTTTGGCCAAGGG 7130

1.3 kb:     74 TGACCTACCATTCCCCTAGTTATGTTTACCACCAATTTGAAAGACGAGCCAAATATAAAA 133
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
J01998:   7131 TGACCTACCATTCCCCTAGTTATGTTTACCACCAATTTGAAAGACGAGCCAAATATAAAA 7190

1.3 kb:    134 GAGAACCCGTCTCACTAACTCTGGCCCTACTATTAGGAGGACTCACTATGGGCGGAATTG 193
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
J01998:   7191 GAGAACCCGTCTCACTAACTCTGGCCCTACTATTAGGAGGACTCACTATGGGCGGAATTG 7250

1.3 kb:    194 CCGCTGGAGTGGGAACAGGGACTACCGCC 222
               ||||||||||||||||||||||||||||
J01998:   7251 CCGCTGGAGTGGGAACAGGGACTACCGCC 7279
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Figure 2. Identification of a positive clone as the murine endogenous ecotropic retrovirus *env* gene **A:** Reactivity of immuno sera with 1.3-kb clone. Sera from each group of immunized mice AS1-antisera from group I mice, AS2-antisera from group II mice, AS3-antisera from group III mice from plates of purified plaques expressing a 1.3-kb portion of the envelope gene (1.3-kb insert) or a negative control (negatibe insert). **B:** Partial sequence analysis of the 1.3-kb clone, which was reactive with sera from all three groups of immunized mice, aligned with the sequence of the murine endogenous ecotropic retrovirus. Approximately 200 bp of the 5′ end of the 1.3-kb clone is shown in alignment with the endogenous ecotropic murine leukemia virus *env* sequence acquired from GenBank (accession number J01998).

homolog of the *ATRX* gene (accession number AF026032). This gene encodes a protein with potential DNA binding and helicase activity.[42,43] The sequence of the 2.8-kb fragment derived from MC38 mRNA was not mutated compared with the published sequence.

Assessment of env and ATRX mRNA expression in murine tumor cells

Northern blot analysis was performed to determine whether the *env* and *ATRX* genes are actively transcribed in MC38 cells (Fig 3). Total RNA was isolated from MC38 cells and several other murine tumor cell lines. These included the C57BL/6-derived cell lines B16 (melanoma) and EL4 (lymphoma) as well as the BALB/c-derived colonic line CT26 and the mammary tumor cell lines 4T1 and 4T07. The mastocytoma cell line P815 from DBA/2 mice was also analyzed, as was the non-transformed myoblast cell line C2C12. Total RNA was also isolated from the normal colonic epithelium of a C57BL/6 mouse. The endogenous ecotropic *env* gene was implicated previously as a melanoma-specific Ag in B16 cells.[44] High levels of *env* gene expression were

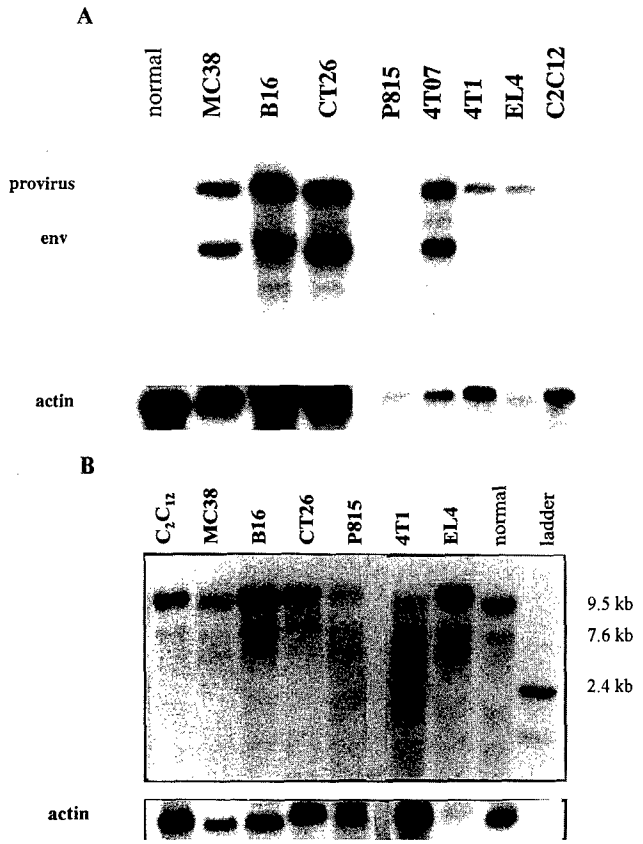

Figure 3. Northern blot analysis of *env* and *ATRX* gene expression in normal colonic epithelium and murine tumor cell lines. **A:** Expression of the *env* gene was examined. Total RNA was hybridized with an env-specific probe. The lower transcript presumably represents the mature, spliced *env* transcript, whereas the upper band represents the full proviral transcript. No expression of the *env* gene was detected in normal colonic epithelium, even after prolonged autoradiography. The blot was stripped and rehybridized with a mouse β-actin probe to demonstrate approximately equal loading **(bottom panel)**. **B:** Expression of the *ATRX* gene was similarly examined. The full-length transcript (~10 kb) and an apparent alternatively spliced form were detected.

detected in MC38, B16, CT26, and 4T07 cells (Fig 3A). Expression was also noted in 4T1 and EL4, with very low levels of expression in P815 cells. Expression of the proviral sequences was not detected in the myoblast line C2C12 or in normal colonic epithelium. Thus, although not expressed in normal cells, the ecotropic endogenous retrovirus is expressed in a variety of murine tumor cell lines, including both spontaneously arising tumors (B16, 4T1, and 4T07) and carcinogen-induced tumors (MC38, EL4, P815, and CT26). The *ATRX* gene was found to be expressed in all of the cell lines tested, as well as in normal colonic epithelium (Fig 3B). Expression was strongest in the tumor cell lines B16 and EL4.

Southern blot analysis

We subsequently investigated the genomic structure of the env-encoding proviral sequence in MC38 cells. Endogenous retroviruses may become activated as the result of a point mutation and remain single copy,[45] or activation may be accompanied by amplification or rearrangement of the proviral sequence.[46] C57BL/6 cells normally contain a single copy of the endogenous ecotropic proviral sequence, which resides on chromosome 8.[47] To determine whether this MuLV proviral sequence was amplified and/or rearranged in MC38 cells, Southern blot analysis was performed. Genomic DNA from normal colon, MC38 cells, and B16 cells was digested with the restriction enzymes *Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II, and *Kpn*I. Hybridization of the DNA with the ecotropic-specific *env* probe revealed the presence of novel fragments in the MC38 DNA; these fragments were not found in the normal DNA and in some cases were distinct from those of B16 DNA (Fig 4). This finding suggests that the MC38 proviral sequence has likely undergone amplification and reintegration into new genomic sites. Southern blot analysis of the *ATRX* gene revealed no apparent genomic rearrangements in MC38 or B16 cells compared with normal genomic DNA (data not shown).

Analysis of the env sequence

We chose to further study the env protein as a tumor Ag in this mouse model to determine the basis of its immunogenicity. To determine whether the env sequence expressed in MC38 cells represents a mutant or nonmutant tumor Ag, we sequenced the expressed *env* sequence and compared it with the published sequence, derived from the AKR mouse strain,[41] and with the endogenous sequence in C57BL/6 genomic DNA. The full-length *env* cDNA was amplified from MC38 total RNA by PCR after reverse transcription. The normal sequence was amplified from genomic DNA extracted from a C57BL/6 mouse. Previously reported coding sequence differences between B16 *env* (also derived from a C57BL/6 mouse) and that of the AKV[44] were either not found in the MC38 expressed sequence (nucleotides 6116, 6419, 7017, and 7085[41]) or found to be the same in the MC38-derived *env* and the C57BL/6 genomic sequence. Thus the differences between the MC38 and AKR-derived sequences likely represent variations between the mouse strains. Also of note, as reported by others,[44,46] the *env* gene expressed in MC38 cells has a 99-bp deletion in the 3′ LTR compared with AKV; however, this change also appeared to be present in the C57BL/6 genomic sequences when size was examined by PCR (data not shown).

Polynucleotide immunization

As both a preliminary and rapid means of assessing the potential of the env polypeptide to act as an immunogenic tumor Ag, the 1.3-kb insert was rescued as phagemid DNA from the reactive plaque and used for polynucleotide immunization. Mice were immunized with the env-encoding DNA, and Ab production was subsequently measured. Intramuscular immunization of C57BL/6 mice with the nonmutant *env* sequence elicited

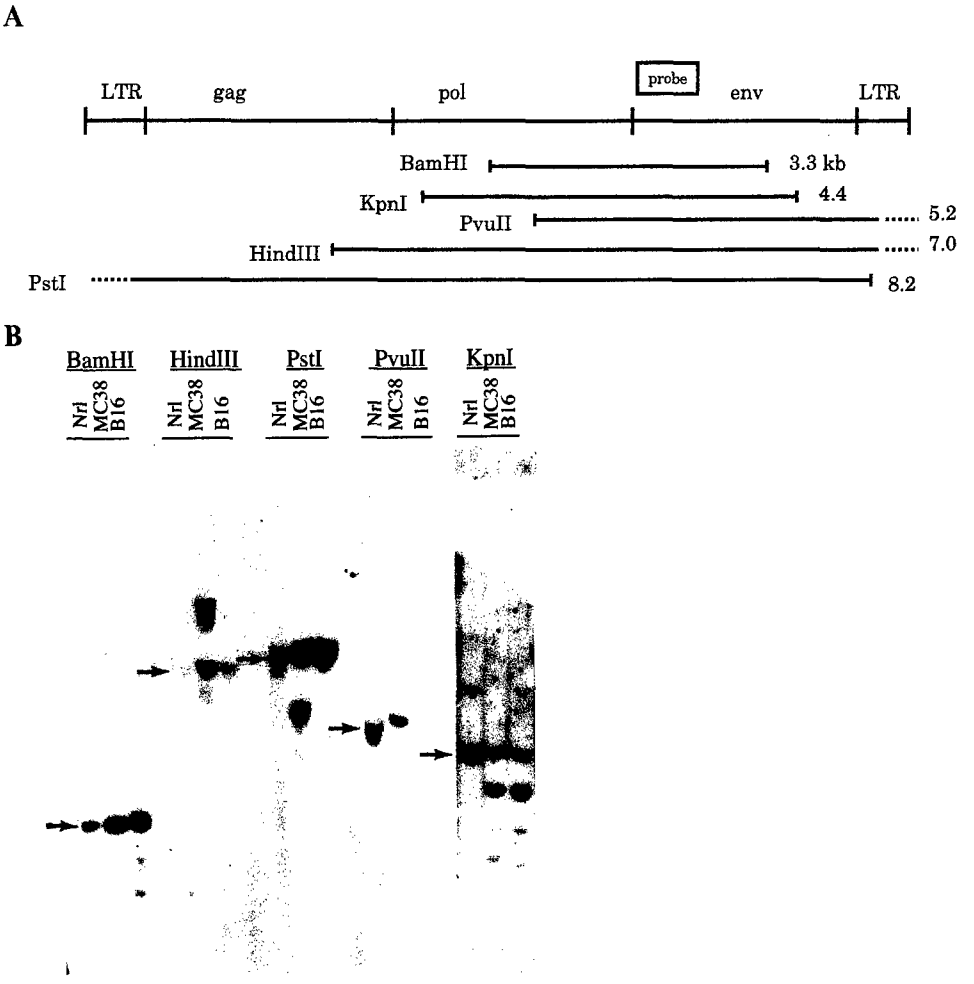

Figure 4. Southern blot analysis of the endogenous ecotropic retrovirus in C57BL/6 genomic DNA, MC38 genomic DNA, and B16 genomic DNA. **A:** Diagrammatic representation of restriction sites in the ecotropic endogenous proviral sequence. The location of the ecotropic-specific probe is indicated. **B:** Genomic DNA from C57BL/6 mouse colon (Nrl), MC38 cells, and B16 cells was digested with the indicated restriction enzymes and hybridized with the ecotropic specific *env* probe. The arrows indicate the location of the endogenous proviral sequence in normal C57BL/6 genomic DNA.

a strong, specific anti-env Ab response in all immunized mice (Fig 5). By contrast, animals immunized with the vector alone exhibited no reactivity with the env protein. Isotype analysis of the anti-env Ab response revealed the presence of several isotypes, including IgG1, IgG2a, and IgG2b, suggesting the presence of both Th1 and Th2 type responses.[48,49]

DISCUSSION

A limited number of murine tumor Ags have been described to date, and the ability of these Ags to elicit effective antitumoral immunity is currently an area of active investigation. Although prospects for the development of immunotherapeutic modalities have recently been improved by advances in the field of immunology,[50,51] knowledge of the spectrum of tumor Ags important in mediating immune interactions is needed. In addition, it is important to catalog the nature of murine tumor Ags, such that a comparison with human cancers can be explored and more appropriate animal models can be developed for evaluating immunotherapeutic approaches to cancer. We have used a strategy for tumor Ag identification that exploits the humoral immune responses that accompany the induction of antitumoral immunity and have identified the MuLV endogenous ecotropic *env* gene-encoded protein and the ATRX protein as candidate tumor Ags in MC38 colon adenocarcinoma cells.

The ATRX protein is expressed in a wide variety of normal tissues in mice and humans.[52] This protein contains highly conserved domains, which classify it as a member of the helicase/adenosine triphosphatase superfamily.[53] It thought to be a nuclear protein involved in the regulation of gene expression.[42,54] In humans, a deficiency in this protein is associated with a severe mental retardation syndrome (ATR-X syndrome) which includes α-thalassemia, urogenital abnormalities, and a characteristic facial appearance. A role for *ATRX* in cancer has not yet been described. Northern blot analysis demonstrates that this gene is expressed to a variable degree in all murine tumor lines tested. No mutations were identified in the partial cDNA isolated, and additional studies are underway to determine the basis of this protein's immunogenicity as well as to evaluate the potential of ATRX to serve as a tumor Ag.

More is known about the role of the env protein as a potential tumor Ag. This protein is encoded by one of the numerous endogenous retroviral sequences present

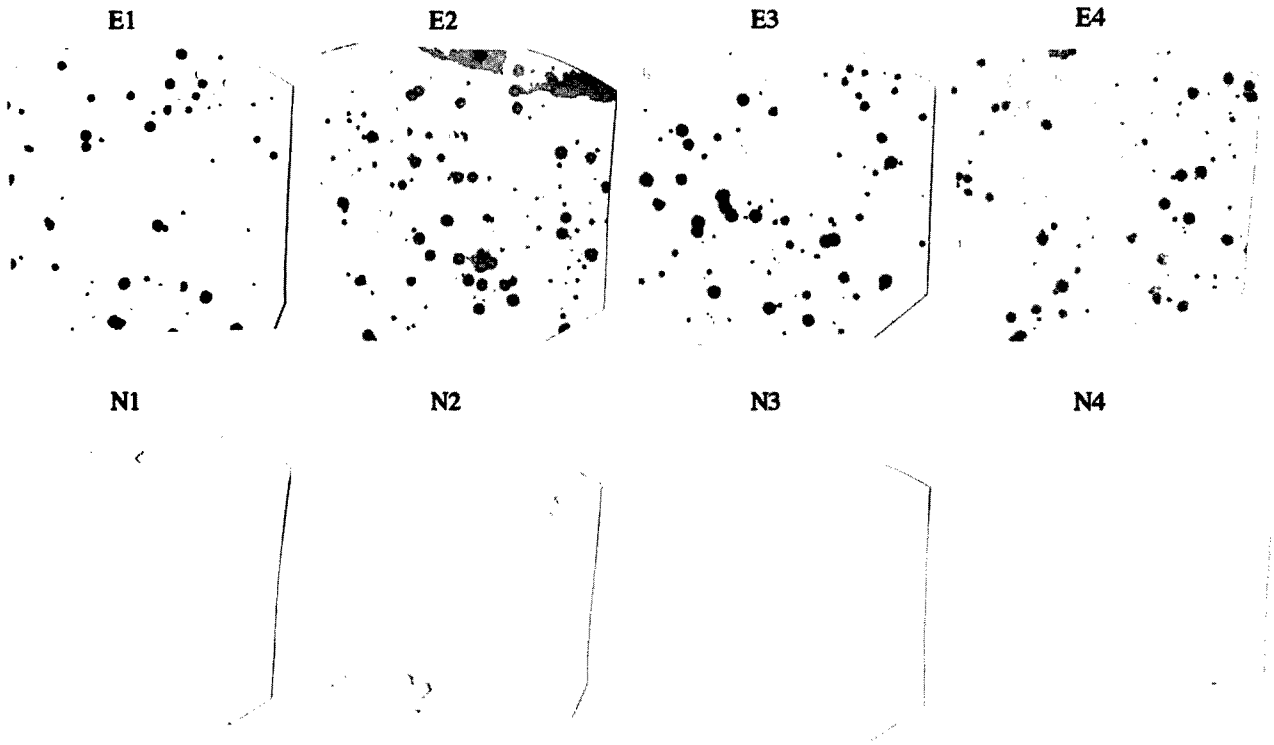

Figure 5. Induction of a humoral immune response to the env protein after polynucleotide immunization. Sera from four mice immunized with the 1.3-kb *env* cDNA (E1-E4) were incubated with filter lifts of phage encoding the env protein mixed with an approximately equal ratio of control, negative phage. Half of the plaques (encoding env) are strongly reactive, demonstrating the specificity of the reaction. Sera from mice immunized with an empty phagemid vector (N1-N4) were used for screening in the same manner, and did not contain Abs that recognize the env protein.

in the normal mouse genome. Endogenous retroviruses are relatively stable elements that are present in multiple copies throughout the genome and inherited in a Mendelian fashion.[55] C57BL/6 mice carry a single copy of an endogenous ecotropic virus, and it is defective and transcriptionally silent in normal tissues.[44] Activation of endogenous retroviruses spontaneously or after exposure to carcinogens has been described in several mouse strains. Activation may occur by several mechanisms, including point mutation,[45] transcomplementation or recombination with other endogenous retroviruses,[56–58] or by amplification and rearrangement of the locus.[46] Here, activation of *env* expression in MC38 cells is associated with the appearance of novel bands on Southern blot analysis, suggesting that amplification and reintegration events are likely to have occurred. The exact nature of the activating mutation(s) will require further study.

Although the env protein was identified in this study on the basis of humoral immunity, it has been determined to be a target of CTL activity in both C57BL/6 mice[44] and BALB/c mice.[59] The identification of a CTL-activating protein by immunoscreening supports the use of Ab-based screening for the identification of relevant tumor Ags. Further, although most murine tumor Ags are thought to be unique to a particular tumor,[60,61] our findings suggest that *env* is expressed in several murine tumors. This Ag is expressed in both spontaneous and mutagen-induced tumor cell lines derived from mice of different genetic backgrounds and from a variety of tissue types (Fig 3A). Sequence analysis revealed no apparent differences between the sequences expressed in the MC38 cells and the genomic sequences of the *env* gene, suggesting that this Ag is a true "self" Ag. Thus, env is immunogenic based on its expression in the tumor. Polynucleotide immunization demonstrated that this protein is capable of acting as an immunogen in C57BL/6 mice. A specific IgG Ab response was elicited in all immunized mice. Interestingly, this Ag may also have relevance to human cancer. The human homologs of the *env* gene, contained in human endogenous retroviral elements (HERVs), may also act as tumor Ags. HERVs are also widely dispersed throughout the human genome. Unlike mice, however, expression of a few of these proviral sequences has been reported in some normal human tissues, most notably placenta.[62] HERV expression has also been noted in a variety of tumor cell lines. Recently, Abs to the HERVK10 env sequence were reported to be present in a large percentage of patients with seminoma.[63] These attributes may make this tumor Ag an ideal target for additional studies relevant to tumor vaccinology.

In summary, application of SEREX to mouse models of carcinoma offers a comparatively rapid means of

identifying putative tumor Ags, which can then be evaluated for utility in gene therapy approaches to cancer immunotherapy. The development of more accurate preclinical models for the evaluation of improved vaccination strategies is one important goal of such studies. This approach may also be important for expanding known human tumor Ags through the identification of murine homologs.

ACKNOWLEDGMENTS

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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